

Amendments to the Specification:

Please replace the paragraph beginning at page 6, line 4, with the following amended paragraph:

--Fig. 1 shows results of treatment with C3 to stimulate neurite outgrowth on inhibitory MAG substrates. Fig. 1A shows PC12 cells plated on MAG remained rounded and did not extend neurites. Fig. 1B shows cells plated on MAG in the presence of C3 grew neurites. Fig. 1C shows PC12 cells plated on polylysine (PLL) substrates as a positive control.--

Please replace the paragraph beginning at page 6, line 8, with the following amended paragraph:

--Fig. 2 shows the role of integrins in overriding growth inhibition by myelin. The anti- $\alpha 1$ integrin function blocking antibody, 3A3, was used to determine if integrin function is necessary for laminin to override growth inhibition by myelin or MAG. For experiments on myelin substrates (Figs. 2A-D), cells were fluorescently labeled with DiI, and plated on myelin (Fig. 2A), polylysine (Fig. 2B), or myelin +1 μ g laminin (Figs. 2C and D). Control IgG was added to samples shown in Figs. 2A-C, the 3A3 antibody to the sample shown in Fig. 2D. Neurites do not extend on myelin but grow on laminin or mixed laminin/myelin substrates. When 3A3 is added, laminin no longer overrides growth inhibition by myelin. [[Panels]] [[Figs. 2E-H]] show by phase contrast cells plated on recombinant MAG (Fig. 2E), laminin (Fig. 2F), or recombinant MAG plus laminin (Figs. 2G and H), with control antibody (Figs. 2E-G) or with 3A3 (Fig. 2H). Integrin function is needed to override growth inhibition by MAG.--

Please replace the paragraph beginning at page 6, line 18, with the following amended paragraph:

--Fig. 3 presents the results of studies in which PC12 cells transfected with dominant negative Rho extend short neurites on MAG substrates. Mock-transfected PC12 cells

([[a,c,e]] Figs. 3A, C, and E) or cells transfected with dominant-negative Rho ([[b,d,f]] Figs. 3B, D, and F) were plated on laminin ([[a,b]] Figs. 3A and B) or MAG ([[c-f]] Figs. C-F). MAG inhibits neurite outgrowth ([[c]] Fig. 3C), but dominant negative Rho cells spread on MAG and some cells extend short neurites ([[d]] Fig. 3D). Treatment with C3 further stimulates neurite outgrowth on MAG from both lines of cells ([[e,f]] Figs. 3E and F).--

Please replace the paragraph beginning at page 6, line 24, with the following amended paragraph:

--Fig.[[ure]] 4 shows activation of Rho on MAG substrates. Activated Rho is associated with the plasma membrane. To determine if activated Rho was detected under conditions where PC12 cells do not grow neurites, cells were grown in suspension or plated on MAG or collagen substrates. Two hours later the plasma membranes were purified, the proteins separated by SDS PAGE, and the proteins transferred to nitrocellulose and stained with Ponceau S (top panel). Rho A was detected on the blots by immunoreactivity with anti-RhoA antibody (bottom panel). Immunoreactivity was strongest when cells were grown in suspension or when cells were plated on MAG. Therefore, Rho A is more active when cells are kept in suspension or plated on MAG than when plated on growth-permissive collagen.--

Please replace the paragraph beginning at page 7, line 3, with the following amended paragraph:

--Fig.[[ure]] 5 shows treatment of retinal neurons with C3 stimulates neurite growth on polylysine and MAG substrates. On nMAG substrates neurite growth is inhibited ([[a]] Fig. 5A), but after C3 treatment retinal neurons plated nMAG substrates extend neurites ([[b]] Fig. 5B). Growth of neurites from retinal neurons plated on PLL ([[c]] Fig. 5C). Bar, 50 μ m.--

Please replace the paragraph beginning at page 7, line 7, with the following amended paragraph:

--Fig. 6 demonstrates ADP-ribosylation of Rho by C3 detected in cultured cells. PC12 cells or retinal neurons were cultured in the presence (+) or absence of C3 (-) for two days. The cells were lysed, and 10 μ g of protein from each sample was separated on a 11% acrylamide gel. The proteins were transferred to nitrocellulose, probed with mouse anti-RhoA antibody and anti-mouse-HRP antibody, and revealed by a chemiluminescent reaction (top panel). The membranes were then reprobed with rabbit anti-Cdc42 and anti-rabbit alkaline phosphatase and revealed with NTB/BCIP color reaction (bottom panel). Treatment of cells with C3 results in an ADP-ribosylation-induced decrease in the mobility of RhoA. The mobility of Cdc42 does not change with C3 treatment.--

Please replace the paragraph beginning at page 7, line 16, with the following amended paragraph:

--Fig. 7 illustrates methods used to study the effect of C3 on injured optic nerve. [[Figure 7a]] Fig. 7A shows the optic nerve was removed from the sheath prior to crushing with 10.0 sutures (top panel) and C3 was applied in Gelfoam and Elvax tubes (rectangular bars in middle and bottom panels) immediately following optic nerve crush (middle panel). The retinal ganglion cell axons were detected by anterograde labeling with cholera toxin and immunodetection of the cholera toxin in longitudinal sections of the optic nerve (bottom panel). Fig. 7C-F show treatment of crushed optic nerve with C3 stimulates regenerative growth of retinal ganglion cells axons. Fig. 7C) Longitudinal 15 μ m section of a buffer-treated control optic nerve showing the failure of RGC axons to cross the injured region; (Figs. 7D and E) Longitudinal 15 μ m sections of two different optic nerves treated with C3 showing anterogradely-labeled axons extending past the crush (arrows). The site of crush is indicated with arrowheads; Fig. 7F) Higher magnification view of (e) in Fig. 2E showing the twisted growth of regenerating axons. Bar, 100 μ m (Figs. 7C-E) and 50 μ m in Fig. 7F. Fig. 7B shows quantitation of axon regeneration across the site of lesion. Representation of regeneration observed in different animals. For each animal, the maximum number of axons observed in a single 14 μ m section was counted at different distances

from the site of the crush. Each point represents one animal, but animals with growth past 500 μm are also represented at the shorter distances. Large numbers of regenerating fibers ($>10/\text{section}$) were observed to cross the lesion after C3 treatment compared to treatment with PBS.--

Please replace the paragraph beginning at page 8, line 3, with the following amended paragraph:

-- Fig. [[ure]] 8 shows the effect of Y27632 on neurite outgrowth of primary neurons plated on inhibitory substrates compared with C3. P0 retinal ganglion cells (RGCs) were examined after 48 hours on the following test substrates: Polylysine (PLL) chondroitin sulfate proteoglycan (CSPG) or myelin in the presence or absence of C3 or Y27632. Shown is the average RGC neurite length after treatment with 50 $\mu\text{g}/\text{ml}$ C3 or Y27632 (dense hatching), 25 $\mu\text{g}/\text{ml}$ C3 or Y27632 (light hatching), or no treatment (white).--

Please replace the paragraph beginning at page 8, line 9, with the following amended paragraph:

-- Fig. [[ure]] 9 shows the measurement of regeneration distances in mice with spinal cord injury alone, in mice treated with collagen and fibrin as controls, in mice animals treated with C3 in collagen or fibrin gels, and in mice treated with Y27632 in fibrin. Each point represents one animal. The circles are animals examined at 3 weeks to one month, the triangles animals examined 3 months after spinal cord injury.--

Please replace the paragraph beginning at page 8, line 14, with the following amended paragraph:

-- Fig. [[ure]] 10 shows an analysis of functional recovery. Modified BBB scores of C3-treated (black circles), Y27632-treated (black triangles), fibrin-treated (hatched circles), and untreated (open circles) mice to evaluate recovery of locomotion for one month following dorsal

over-hemisection. Each point represents the average of 10-11 animals \pm SEM for controls and C3 experiments, or 5 animals for Y27632.--

Please replace the paragraph beginning at page 25, line 20, with the following amended paragraph:

--To explore the possibility that treatment of damaged axons with C3 might foster regeneration *in vivo*, we examined regeneration of retinal ganglion cell (RGC) axons in the optic nerve 2 weeks after optic nerve crush. Recently, it has been shown that microlesions in the CNS reduce the extent of the glial scar and allow axons access to CNS white matter distal to the lesion (Davies, S.J.A., *et al.* (1997) *Nature* 390, 680-683). To make microlesions of optic nerve, 10.0 sutures were used to axotomize RGC axons by constriction (Fig. 7[a]A). Retrograde labeling of RGCs from the superior colliculus (not shown), as well as anterograde labeling techniques (eg., Fig 7[a]A) verified that RGC axons were effectively axotomized. To apply C3 to crushed nerves, Gelfoam soaked with 2 mg/ml C3 was wrapped around the left optic nerve at the crush site, and two Elvax tubes, each loaded with 20 mg of C3, were positioned for sustained slow release (Fig. 7[a]A). Twelve animals were treated with C3 and a further 8 animals were treated with PBS as controls. Crushed and regenerating axons were visualized by anterograde labeling with cholera toxin injected into the eye 12 days after optic nerve crush (Fig. 7[a]A). Fourteen days after optic nerve crush, longitudinal cryostat sections of the optic nerves were examined by fluorescent microscopy for immunoreactivity to cholera toxin to detect anterogradely labeled RGC axons.--

Please replace the paragraph beginning at page 26, line 4, with the following amended paragraph:

--In control optic nerves that received optic nerve crush alone, no RGC axons extended past the crush site (n= 3 animals). In control animals treated with PBS-Elvax pellets and gelfoam, the crush site was easily detected where most anterogradely labeled axons stopped

abruptly (Fig. 7[c]C). However, in these animals, a few axons did extend past the crush (Fig. 7[c]C, arrows), and the numbers of axons that regenerated varied from animal to animal. The application of Gelfoam and Elvax tubes may have altered the response to injury. Nonetheless, the response to C3 treatment applied with this lesion paradigm was dramatic.--

Please replace the paragraph beginning at page 26, line 11, with the following amended paragraph:

--We observed that C3 treatment allowed many RGC axons to grow past the region of the lesion. In 7 of 12 C3-treated animals, the lesion site was not clearly defined because of the large numbers of axons that extended through the site (Figs. 7[d and e]D and E). Many of the axons that extended past the lesion site showed a twist path of growth, supporting their identification as regenerating axons (Fig. 7[f]F). A quantitative comparison of C3 and PBS treated animals revealed that more fibers grew past the lesion site after C3 treatment than after PBS treatment (Fig. 7[b]B). For this analysis we made a conservative estimate of the lesion site based on morphology, and counted the number of fibers in the distal optic nerve in 14 μ m sections. Seven of 12 C3-treated animals showed at least one section with 10-20 axons extending 250 μ m past the crush, compared with 1 of 8 of the PBS-treated controls (Fig. 7). In some animals regenerating axons were observed up to 1 mm from the crush, an extent of regeneration similar to that observed in mouse optic nerve after treatment with IN-1 antibody to block myelin inhibitors where fibers extended up to 750 μ m (Bartsch, U., *et al.*, (1995) *Neuron* 15, 1375-1381).--

Please replace the paragraph beginning at page page number, line line number with the following amended paragraph:

--Rats were anesthetized with 0.6 ml/kg hypnorm, 2.5 mg/kg diazepam and 35 mg/kg ketamin. The left optic nerve was exposed by a supraorbital approach, the optic nerve sheath slit longitudinally, the optic nerve lifted out and crushed 1 mm from the globe by constriction with a 10.0 suture held for 60 seconds (Fig. [4a]7A). For C3 treatment and buffer controls, Gelfoam

soaked in PBS or 2mg/ml C3 transferase was placed on the nerve at the lesion site. Two 3 mm long tubes of Elvax (Sefton, *et al.*, (1984)) loaded with buffer or 20 mg C3 were inserted in the Gelfoam near the nerve for continued slow release of C3 (Fig. [4b]7A). Twelve days after crush, 5 ml of 1% cholera toxin β subunit (List Biological laboratories, Inc., Cambell, CA) was injected into the vitreous to anterogradely label retinal ganglion cell axons (Fig. [4c]7A). Two weeks after optic nerve crush the animals were fixed by perfusion with 4% paraformaldehyde, and the eye with attached optic nerve was removed and postfixed in 4% paraformaldehyde. Longitudinal cryostat sections were processed for immunoreactivity to cholera toxin with goat anti-cholera toxin at 1:12,000 (List Biol. Labs Inc., CA) followed by rabbit anti-goat biotinylated antibody (1:200, Vector Labs, Burlingame, CA), and DTAF-streptavidin (1:500, Jackson Immunoresearch Laboratories).--

Please replace the paragraph beginning at page 29, line 29, with the following amended paragraph:

-- Neurons plated on chondroitin sulfate proteoglycans (CSPG) or purified myelin had a rounded shape. After treatment with C3 or Y27632, neurons plated on complex inhibitory substrates were able to extend neurites. Treatment either with C3 or Y27632 significantly increased the length of neurites compared to untreated cells plated on myelin or CSPG. These results demonstrate that inactivation of Rho or inhibition of ROK stimulates retinal neurons to extend neurites on growth inhibitory substrates. These results, illustrated in Fig. [[ure]] 8, were analyzed quantitatively by measuring the average of retinal ganglion cells neurite length of the longest neurite per cell after 48 hours on PLL, CSPG or myelin including C3 or Y27632.--

Please replace the paragraph beginning at page 31, line 3, with the following amended paragraph:

-- To assess the potential of Rho inactivation to treat spinal cord injury (SCI), we cut the spinal cord of adult mice at T7 by a dorsal over-hemisection (Huang et al., Neuron 24:639-647,

1999). We tested local delivery of C3 in collagen (Joosten, J. Neurosci. Res. 41:481-490, 1995) or in a fibrin adhesive (Herbert, 1998) that polymerizes *in vivo* several seconds after injection (Herbert, J. Biomed. Mater Res. 40:551-559, 1998) ; Y27632 was tested in the fibrin adhesive. Anterograde tracing with WGA-HRP of corticospinal tract (CST), a tract often used to study histological regeneration, was used to assess fiber growth in six groups of animals: animals treated with fibrin plus C3 ($n=13$), collagen plus C3 ($n=12$), fibrin plus Y27632 ($n=5$), fibrin alone ($n=10$), collagen alone ($n=7$), and SCI with no treatment ($n=13$) (Figure 9). Without C3 or Y27632 treatment, transected CST axons retracted back from the site of lesion by approximately 300 μm , although in animals treated with fibrin alone some regenerative sprouts did extend from the retracted bundle. Application of C3 to the injured spinal cord elicited extensive sprouting into the dorsal white matter and the lesion scar. Treated animals with Y27632 showed regenerative sprouting into the dorsal white matter and toward the lesion site. To assess axons distal to the lesion site, the distance of the longest axon was measured. Axons were found up to 12 mm from the lesion site in C3 treated animals and up to 3 mm from the lesion site in Y27632 treated animals (Figure 9), while buffer-treated animals showed retraction from the lesion site. Therefore, after treatment with C3 or with Y27632, axons were found to extend past the lesion into the distal white matter. These axons have a twisted course of growth typical of regenerated axons.--

Please replace the paragraph beginning at page 32, line 25, with the following amended paragraph:

-- Twenty-four hours after surgery, control mice were paraplegic (Figure 10) and moved by pulling themselves forward with their forelimbs. Mice treated with C3 or with Y27632 showed a remarkable recovery within 24 hours (Fig. [[ure]] 10), already walking with weight support (Figure 10). While this early recovery is too rapid to be explained by long distance regeneration, possible mechanisms include local reorganization of central pattern generator circuitry (Ribotta et al., J Neurosci 20:5144-52, 2000), pharmacological activation of neurotransmitter receptors (Rossignol et al., Humana Press, Totowa. 57-87, 2000) or

neuroprotection (Laufs et al., J Clin Invest 106:15-24, 2000; Trapp et al., Mol Cell Neurosci 17:883-94, 2001). Mice that had received C3 or Y27632 treatment continued to recover over the 1 month period of observation, and exhibited hindlimb-forelimb coordination. By contrast, the average recovery plateau for untreated animals was limited to unstable walking without hindlimb-forelimb coordination. Retransection of the spinal cord at 3 weeks ($n=8$) eliminated any achieved hindlimb recovery in both C3 treated ($n=5$) and control ($n=3$) animals (data not shown).--